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(71) Applicant *(for all designated States except US)*: IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE [GB/GB]; Sherfield Building, Exhibition Road, London SW7 2AZ (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: MASKELL, Duncan, John [GB/GB]; 6 Sterne's Way, Stapleford, Cambridge CB2 5DA (GB). DOUGAN, Gordon [GB/GB]; Imperial College of Science, Technology & Medicine, Sherfield Building, Exhibition Road, London SW7 2AZ (GB).

(74) Agent: MALLALIEU, Catherine, Louise; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).

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(54) Title: MUTANT *msbB* or *htrB* GENES

(57) Abstract

Nucleic acid for a mutant *msbB* or *htrB* gene derivable from *Salmonella* which results in loss of an *msbB* or *htrB* encoded protein, respectively, or loss of function of the protein, which in turn results in a lipid A molecule having reduced toxicity.

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Mutant msbB or htrB genes

The present invention relates to nucleic acid for a mutant *msbB* gene or a mutant *htrB* gene, a recombinant DNA construct comprising the nucleic acid, a micro-organism comprising a mutant *msbB* or *htrB* gene, an inactivated *msbB* or *htrB* gene or lacking a *msbB* or *htrB* gene, and uses thereof, particularly, but not exclusively, its use in a vaccine.

Lipopolysaccharide (LPS) forms the outer leaflet of the outer membrane of Gram negative bacteria. In most cases it is highly biologically active, being both immunogenic and the active principle in endotoxin. The structure of LPS may be considered to be divided into several domains. The domain of LPS which is required for the activities associated with endotoxin is lipid A. Lipid A molecules are able to induce the release of a number of cytokines as well as nitric oxide and it is through these mediators that the effects of endotoxin are seen.

Most lipid A molecules may be divided into hydrophilic and hydrophobic domains. The hydrophilic region consists of a 1-6 linked D-glucosamine (GlcN) disaccharide backbone substituted by phosphate groups at positions 1 and 4', which may in turn be linked to, or replaced by, pyrophosphorylethanolamine or 4-amino-4-deoxy-L-arabinose. The hydrophobic region consists of fatty acids and these may vary between species. In *S. typhimurium* the lipid A has a fatty acylation pattern in which the 2 and 2' amino groups and the 3 and 3' hydroxyl groups on the diglucosamine are each linked to 3-hydroxytetradecanoic acid (3-OH- 14:0). The 2'-linked fatty acid is further substituted at the 3-hydroxyl group by dodecanoic acid (12:0) and the 3' fatty acid is again further substituted at the 3-hydroxyl group by tetradecanoic acid (14:0) (1,2). These have been called secondary acylations and will hereinafter be referred to as such.

It is believed that the pattern of fatty acylation in natural lipid A may have consequences for the biological activity (and thus toxicity) of the molecule. For example *Rhodobacter sphaeroides* lipid A is non-toxic and differs from toxic lipid A only in the pattern of fatty acyl substitutions (1,2). It is also known that treatment of

lipid A with hydroxide ion cleaves the secondary acyl chains from the molecule with consequent detoxification. Indeed an acyloxyacyl hydrolase is present in neutrophils that catalyses precisely this cleavage and is probably one of the mechanisms responsible for detoxifying lipid A *in vivo*. However, both these systems are naturally occurring and there is no indication of how these observations could be applied to other systems.

Recently Raetz et al. (1,2) have made progress in understanding lipid A biosynthesis in *E. coli*, and have cloned, sequenced and mutagenized most of the genes necessary for it. The early stages of the pathway have been known for some time but the enzymes and genes required for the secondary acylations have only recently been discovered. The addition of secondary fatty acids to the hydroxyl groups of the 2'- and 3'-linked hydroxytetradecanoic acids completes lipid A biosynthesis and these reactions are catalysed by the products of the ***htrB*** and ***msbB*** genes. Very recently Somerville et al. (3) disclosed an *msbB* mutant of *E. coli* which was shown to have greatly reduced ability to induce cytokines (especially TNF alpha) and E-selectin expression in an *in vitro* system. Clearly, phenomena observed using *in vitro* models may have little significance *in vivo*. Further this work is limited to *E. coli*.

EP-A-0 650 733 describes an attenuated vaccine for avian species comprising a micro-organism which may be *Salmonella* or *E. coli* amongst others. The approach taken is to use a micro-organism which exhibits auxotrophy to one or more growth factors, such that it is incapable of growing on a minimal medium in the absence of said one or more growth factors.

We have taken a different approach to the problem of providing vaccines against virulent organisms. We employ an *msbB* mutant gene and/or ***htrB*** mutant gene, gene deletion and/or inactivation to give rise to a bacterium which makes a lipid A molecule which has reduced toxicity or which is non-toxic compared to the

lipid A molecule produced by a wild-type bacterium. This approach has a substantial advantage over the approach described in, for example; EP-A-0 650 733 not least because it results in a vaccine which has a reduced endotoxicity and therefore a reduced reactogenicity.

According to one aspect of the present invention there is provided nucleic acid for a mutant *msbB* gene derivable from *Salmonella* which results in loss of MsbB protein or loss of function of the protein, which in turn results in a lipid A molecule having reduced toxicity compared to the wild-type lipid A molecule.

According to another aspect of the present invention there is provided nucleic acid for a mutant *htrB* gene derivable from *Salmonella* which results in loss of HtrB protein or loss of function of the protein, which in turn results in a lipid A molecule having reduced toxicity compared to the wild-type lipid A molecule.

In other words during the biosynthesis of a lipid A molecule the mutant *msbB* or *htrB* gene results in loss of MsbB or HtrB protein respectively, which in turn results in the biosysnthesis of a lipid A molecule with a reduced ability to induce cytokines.

The lipid A molecule is one which forms part of LPS. Whilst not wishing to be bound by any theory it is believed that the loss of the *msbB* encoded protein or the loss of function of the *msbB* encoded protein will give rise to a lipid A molecule lacking at least secondary acylation of the hydroxyl group of the 2'-linked hydroxytetradecanoic acid of the lipid A. Similarly, it is believed that the loss of the *htrB* encoded protein or loss of function of the HtrB protein will give rise to a lipid A molecule lacking at least secondary acylation of the hydroxyl group of the 3'-linked hydroxytetradecanoic acid of the lipid A molecule.

Thus, preferably the lipid A is deficient in at least one of the secondary acyl chains which are usually associated with a lipid A domain of a lipopolysaccharide. In a particularly preferred embodiment the lipid A molecule lacks both secondary acyl chains.

In one embodiment the mutant is derivable, or derived, from *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Yersinia*, *Vibrio*, *Aeromonas*, *Pasteurella*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Flavobacterium*, *Bordetella*, *Actinobacillus*, *Neisseria*, *Brucella*, *Haemophilus* or *Escherichia coli*.

As previously mentioned the mutant may be derivable, or in a particularly preferred embodiment is derived, from *Salmonella*. Thus the mutant can be arrived at by mutating a wild type *Salmonella* micro-organism or more specifically its *msbB* or *htrB* gene. However, synthetic nucleic acid fall within the scope of the present invention. Thus although the original mutant may have been derived by mutating *Salmonella*, the mutant may be sequenced and the nucleic acid of interest reproduced, e.g. synthetically, using techniques well known to the skilled worker. This is also true when the mutant is derived from a micro-organism other than *Salmonella*.

Any convenient technique which is, or becomes available, may be used to modify the gene. These will be known to workers skilled in the art. One preferred method uses genetic manipulation of *msbB* or *htrB* by insertion of a kanamycin resistance cassette to inactivate the gene, conjugation of the inactivated gene into the recipient to be mutated on a suicide vector, followed by P22 transduction into other recipients.

In a particularly preferred embodiment of the present invention the micro-organism is *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella paratyphi* A or C, *Salmonella schottmulleri*, *Salmonella choleraesuis*, *Salmonella montevideo*, *Salmonella newport*, *Salmonella enteritidis*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella abortusovi*, *Salmonella abortus-equi*, *Salmonella dublin*, *Salmonella sofia*, *Salmonella havana*, *Salmonella bovis-morbificans*, *Salmonella hadar*, *Salmonella arizonae* or *Salmonella anatum*.

In an especially preferred embodiment of the present invention the micro-organism is *S. typhimurium*, and preferably the strain is C5, SL1344 or HWSH.

Preferably the mutation or loss of protein is not lethal for growth of a micro-organism. This has the advantage that the micro-organism can be easily cultured without having to add supplements to the medium. In fact it is surprising that viable bacterial are produced after alteration of a component of the lipid A molecule.

As previously mentioned, preferably the lipid A molecule has a reduced ability to induce a cytokine response. Ability to induce a cytokine response is a conventional toxicity measure. In general we have found that the lipid A molecules produced by the present invention have the ability to reduce cytokine induction down to about ¼-½ of that induced by wild-type lipid A molecules. More preferably the lipid A molecule and/or micro-organism induces less TNF-α and/or less IL-1β and/or less NO. More preferably the lipid A molecule induces at least 5-fold less TNF-α and at least half as much IL-1β as the corresponding wild-type. In another preferred embodiment the lipid A molecule induces at least half as much NO as the corresponding wild-type. Thus it will be appreciated that the present invention provides for the toxicity to be substantially reduced. In an especially preferred embodiment there is substantially no toxicity.

In one preferred embodiment, the micro-organism of the present invention kills a BALB/c mouse when the population of the micro-organism in the liver and/or spleen reaches about 10^9 per organ. In fact it actually only kills a proportion of the infected mice, around 5-10%, even at such a high level of 10^9 per organ. This can be compared to the wild-type where a micro-organism population of about 10^8 per organ is sufficient to kill all mice infected.

It is preferable to compare the reduced toxicity of the lipid A molecule arrived at using the present invention and/or toxicity of the micro-organism of the present invention against the toxicity of a lipid A molecule produced by the parent

wild-type. By parent wild-type we mean the micro-organism from which the mutant was derived, e.g. the wild-type micro-organism which was used to produce the mutant, or the wild-type micro-organism in which the mutant was engineered.

Thus according to one preferred embodiment of the present invention there is provided nucleic acid derived from *Salmonella* and encoding for a mutant *msbB* gene or a mutant *htrB* gene which results in a lipid A molecule having reduced toxicity compared to the lipid A molecule produced by the respective *msbB* encoded protein or *htrB* encoded protein encoded for by the corresponding *Salmonella msbB/htrB* gene from which the mutant is derived.

Whilst not wishing to be bound by any theory the mutant *msbB* and *htrB* genes of the present invention may result in a polypeptide which is truncated with respect to the polypeptide encoded by the non-mutated gene, or indeed loss of the peptide.

That being said the present invention also encompasses any polypeptide molecule encoded for by the nucleic acid of the present invention and/or produced by the micro-organism of the present invention.

According to a further aspect of the present invention there is provided a recombinant DNA construct comprising the DNA of the present invention cloned into a cloning or expression vector. According to a further aspect of the present invention there is provided a recombinant micro-organism comprising the recombinant DNA construct of the present invention.

It will be appreciated that as well as using a mutant *msbB* or *htrB* gene the same effect may be achieved by actually deleting the *msbB* or *htrB* gene from the genome or by inactivation.

Any convenient technique which is, or becomes available may be used to delete or inactivate the gene. These will be known to workers skilled in the art.

Thus, according to one aspect of the present invention there is provided a

Salmonella micro-organism comprising a mutant *msbB* or *htrB*, an inactivated ***msbB*** or ***htrB*** gene or lacking a ***msbB*** or ***htrB*** gene and having reduced toxicity compared to the parent wild-type, i.e. the *Salmonella* micro-organism from which it is derived.

According to a preferred embodiment there is provided a recombinant *Salmonella* micro-organism transformed with a mutant *msbB* or ***htrB*** gene, comprising an inactivated gene or from which the gene has been deleted and having reduced toxicity compared to the micro-organism prior to transformation.

Preferably the *Salmonella* micro-organism is *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella paratyphi* A or C, *Salmonella schottmulleri*, *Salmonella choleraesuis*, *Salmonella montevideo*, *Salmonella newport*, *Salmonella enteritidis*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella abortusovi*, *Salmonella abortus-equi*, *Salmonella dublin*, *Salmonella sofia*, *Salmonella havana*, *Salmonella bovis-morbificans*, *Salmonella hadar*, *Salmonella arizonae* or *Salmonella anatum*.

More generally, the present invention provides a micro-organism comprising a mutated *Salmonella* ***msbB*** or ***htrB*** gene, an inactivated *msbB* or ***htrB*** gene or a micro-organism from which the ***msbB*** or *htrB* gene has been deleted.

Also the present invention provides a micro-organism comprising an inactivated ***msbB*** or ***htrB*** gene; a mutated *Salmonella msbB* or *htrB* gene or from which the gene has been deleted, and which results in loss of an ***msbB*** encoded protein or ***htrB*** encoded protein, respectively; or loss of function of the protein, which in turn results in a lipid A molecule having reduced toxicity.

According to a preferred embodiment of the present invention the micro-organism is *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Serratia*, ***Proteus***, *Yersinia*, *Vibrio*, *Aeromonas*, *Pasteurella*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Flavobacterium*, *Bordetella*, *Actinobacillus*, *Neisseria*, *Brucella*, *Haemophilus* or

Escherichia coli.

In a particularly preferred embodiment of the present invention the micro-organism is *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella paratyphi* A or C, *Salmonella schottmulleri*, *Salmonella choleraesuis*, *Salmonella montevideo*, *Salmonella newport*, *Salmonella enteritidis*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella abortusovi*, *Salmonella abortus-equi*, *Salmonella dublin*, *Salmonella sofia*, *Salmonella havana*, *Salmonella bovis-morbificans*, *Salmonella hadar*, *Salmonella arizonae* or *Salmonella anatum*.

In an especially preferred embodiment of the present invention the micro-organism is *S. typhimurium*, and preferably the strain is C5, SL1344 or HWSH.

According to yet another aspect of the present invention there is provided a live vaccine comprising an attenuated or avirulent micro-organism having a mutated *msbB* or *htrB* gene, inactivated *msbB* or *htrB* gene or lacking the gene and having reduced toxicity in accordance with the present invention.

The mutation may be introduced into live attenuated vaccine strains of, e.g. *Salmonella*, thus reducing their endotoxicity and thereby reducing their reactogenicity. This would generate safer vaccine strains that would be more acceptable to the licensing authorities and to the general public. The same strategy might be used for all live attenuated Gram negative bacterial vaccines. A prime example here would be the new live attenuated *Shigella* vaccines. The same effect may arise with an inactivated or deleted gene.

According to a further aspect of the present invention there is provided a method of immunising a subject comprising administering a vaccine of the present invention.

Preferably the vaccine is against infection caused by a micro-organism which is *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Yersinia*, *Vibrio*,

Aeromonas, *Pasteurella*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Flavobacterium*, *Bordetella*, *Actinobacillus*, *Neisseria*, *Brucella*, *Haemophilus* or *Escherichia coli*. In a more preferred embodiment the micro-organism is *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella paratyphi* A or C, *Salmonella schottmulleri*, *Salmonella choleraesuis*, *Salmonella montevideo*, *Salmonella newport*, *Salmonella enteritidis*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella abortusovi*, *Salmonella abortus-equi*, *Salmonella dublin*, *Salmonella sofia*, *Salmonella havana*, *Salmonella bovis-morbificans*, *Salmonella hadar*, *Salmonella arizonae* or *Salmonella anatum*.

The subject may, for example, be a mammal or avian. Examples of such mammals include humans, cattle, swine and ovine species. Examples of such avians include chickens, ducks, turkeys, geese, bantams, quail and pigeons.

In order to prepare the vaccine of the present invention the micro-organism must be attenuated or rendered avirulent.

The vaccine composition of the present invention may be administered by injection or orally, and the composition must be suitable for the desired administration route. Suitable vaccine compositions are well known to those skilled in the art.

Bacteria with mutations in the *msbB* or *htrB* gene, an inactivated gene or lacking the gene would provide excellent background strains for the production of proteins and nucleic acid for vaccines and therapeutics, substantially removing the requirement for downstream processing to remove the erstwhile toxic LPS molecules.

Thus according to yet another aspect of the present invention there is provided use of a micro-organism having a mutant *msbB* or *htrB* gene, an inactivated gene or lacking said gene and having reduced toxicity in the recombinant production

of a protein or gene of interest.

The isolated LPS made by these mutants may be useful as an endotoxin antagonist.

A sample of *S. typhimurium* C5 having a *msbB* mutation in accordance with the present invention has been deposited under the Budapest Treaty at NCIMB on 17 January 1997 and accorded the deposit number NCIMB 40856. Further characteristics of this deposited micro-organism are given below in the Examples.

Mutants lacking both *htrB* and *msbB* may synthesise Lipid IV_A - KDO_2 which is a non-toxic antagonist of lipid A. These mutants will thus be a source of this molecule which may be used to treat septic shock resulting from endotoxaemia. Thus it will be appreciated that the present invention also extends to constructs and micro-organisms comprising (i) a mutant *msbB* gene which results in loss of MsbB protein or the loss of function of the protein; an inactivated *msbB* gene; or which lacks the *msbB* gene, in combination with (ii) a mutant *htrB* gene which results in loss of HtrB protein or loss of the function of the protein; an inactivated *htrB* gene; or which lacks the *htrB* gene. The present invention also includes the use of such a so-called *msbB/htrB* double mutant as a vaccine and pharmaceutical compositions comprising it, together with its use in producing genes and proteins of interest.

Preferably the mutations in accordance with the present invention are mutations which are substantially incapable of reversion. A substantially non-reversible mutant has a reversion frequency preferably of $\leq 10^{-8}$, more preferably $\leq 10^{-9}$, even more preferably $\leq 10^{-10}$, and most preferably a mutant with zero reversion.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example and with reference to the accompanying drawings in which:

Figure 1 is a graph showing growth curves of wild-type and *msbB* mutant *S.*

typhimurium in BALB/c mice. The two growth curves are indistinguishable in the first week of infection. All the mice infected with wild-type organisms died by 1 week post-infection, whereas most of the mice infected with the *msbB* mutant survived. Subsequently the *msbB* mutant was cleared from the livers and spleens of infected animals;

Figures 2a and 2b are graphs representing the *in vitro* analysis of TNF-α and IL-1β. $2x10^6$ cultured J774 macrophage-like cells were incubated with 10^5 *msbB* mutant or wild-type *Salmonella* both of which had been heat-killed. A time course of release of TNF-α and IL-1β from these cells in response to the bacteria was determined. Mutant *Salmonella* induce 5-fold less TNF-α (Figure 2a) and half as much IL-1β (Figure 2b) as the wild-type organism;

Figure 3 is a graph representing NO generation *in vitro*. $2x10^6$ cultured J774 macrophage-like cells were incubated with 10^7 *msbB* mutant and wild-type *Salmonella* that had been heat-killed. Following 24 hours incubation, the culture medium was assayed for NO by the Griess reaction, which detects NO by determining nitrate/nitrite in the medium. Mutant *Salmonella* induced half as much NO as wild-type bacteria;

Figures 4a and 4b are graphs representing an *in vivo* study of cytokines. Serum samples were taken at 24 hours from mice infected with wild-type or *msbB* mutant organisms. These samples were assayed for TNF-α (Figure 4a) and IL-1β (Figure 4b) by ELISA. Analysis of the results showed that the mutant was inducing approximately 4-fold less TNF-α and 2-fold less IL-1β than the wild-type bacteria. These results correlate precisely with the observed reduced lethality of the mutant;

Figure 5 is a graph representing the results of Example 3, an oral vaccination study using an *aroA* mutant of *S. typhimurium* in BALB/c mice; and

Figure 6 is another graph representing the results of Example 3, an oral

vaccination study using an *msbB/aroA* mutant of *S. typhimurium* in BALB/c mice.

Whilst not wishing to be bound by any theory we believe that the fatty acyl substitutions in a lipid A molecule of the LPS domain of a bacterium, to a large extent, determine the toxicity of the molecule and, furthermore, if alterations in fatty acid substitution could be engineered, then previously toxic LPS molecules may be detoxified. Thus, genetic manipulation of bacteria such that at least one of the secondary acyl chains is not added should reduce the molecule's toxicity and furthermore engineering a bacterium such that both (or all) secondary acyl chains are not added should make the lipid A molecule substantially non-toxic.

Work using natural *Salmonella* infections in mice to assess the precise role of lipid A and endotoxin in disease is described below.

Salmonella typhimurium causes a severe invasive disease in mice, which shares many features in common with typhoid fever, caused by *S. typhi* in humans. Mouse typhoid has been extensively investigated, generating a vast amount of data regarding virulence and immunogenicity (4). Using parenteral inoculation into inbred mice, several patterns of growth of the bacteria *in vivo* have been observed, and this growth is controlled by a number of host genetic systems. The best studied of these is that regulated by the *Ity* gene, which has recently been cloned and named *nramp*. After intravenous inoculation of *S. typhimurium* into mice over 90% of the inoculum is killed within the first few hours of infection, but the survivors then live and grow within macrophages of the mononuclear phagocyte system (MPS). The rate of growth of the bacteria over the first few days of infection is controlled by *nramp* such that inbred mice may be divided into susceptible and resistant types. Susceptible mice (e.g. BALB/c) allow the growth of typical virulent Salmonellae at a rate of increase of about ten-fold per day per liver or spleen. Resistant mice (e.g. A/J) allow only half this rate of increase.

In lethal infections the Salmonellae grow too fast to be controlled by primary

host responses and rapidly reach levels of approximately 10^8 per organ. The mice then die with extensive organ damage. The precise mechanism of tissue damage leading to death in mouse typhoid is unknown. It has been speculated that endotoxin levels in the livers, spleens and other infected organs become so high that tissue is damaged, but there is currently no direct evidence to support this. Tissue damage may be induced, in the presence of high levels of endotoxin, by cytokine-mediated responses.

In sublethal infections bacterial growth in the livers and spleens is suppressed around day 4 to 5 of infection by a T-cell-independent mechanism and bacterial counts reach a "plateau". This requires recruitment of bone marrow-derived cells and coincides with the formation of focal lesions then granulomas. The induction of plateau requires the involvement of a number of cytokines, including TNFα and IFNγ(5-7). TNFα acts to recruit monocytes to the site of infection and IFNγ is required for macrophage activation. An IL-12 requirement in this induction has also recently been established, with it probably acting as a positive modulator of IFNγ production (8). LPS, and more specifically its lipid A domain, has been described as a potent inducer of all three of these cytokines in many systems. It is possible that the signal inducing the host to begin synthesising these cytokines, and subsequently to control the infection in mouse typhoid, is dependent, at least in part, on the lipid A domain of LPS. This hypothesis has not been established previously for this model.

An intriguing feature of immunisation of mice with live attenuated *S. typhimurium* vaccine strains is that 7 days after immunisation of C3HeB/FeJ mice there is profound suppression of responses to B- and T-cell mitogens and suppression of the capacity of spleen cells to mount primary *in vitro* plaque-forming-cell responses to sheep erythrocytes. This inhibition is mediated by nitric oxide (9,10). The bacterial inducer of the nitric oxide response leading to

immunosuppression in this model is unknown, but it is likely that endotoxin is involved.

In summary, in mouse typhoid it seems probable that low-level induction of cytokine responses by low levels of LPS, likely to be present in sublethal infections, induces a protective mechanism that enables the animal to survive (i.e. plateau) while the tissue responses induced by the high levels of LPS likely to be present in the last stages of a lethal infection are damaging and eventually lead to organ failure and death.

Example 1

We have cloned and partially sequenced the *msbB* gene from *S. typhimurium*. Briefly, a probe based on *E. coli msbB* DNA sequence was generated using the polymerase chain reaction (PCR), cloned and radiolabelled. This was used to probe a Southern blot of *Salmonella typhimurium* DNA, identifying a 3.2kb *Dra*I fragment. In addition the oligonucleotides were also used in a PCR using *S. typhimurium* DNA as template. This generated an approximately 1kb piece of DNA which was cloned into pGEM-T. On sequencing from either end of this construct it was clear from amino acid and DNA sequence homology that this was *msbB*. To generate an *msbB* mutant in the *S. typhimurium* chromosome it was first necessary to insert an antibiotic resistance marker into the *msbB* coding sequence. To do this new oligonucleotides, based on the *Salmonella* DNA sequence, were generated and used to PCR the gene from the pGEM-T clone. This was then treated with Klenow enzyme to blunt the ends of the DNA and digested with *Sal*I to cut the DNA into 450bp and 550bp fragments. The *Sal*I site is in the coding sequence of the *Salmonella msbB* gene. A gene cassette encoding kanamycin resistance (Pharmacia) was also cut with *Sal*I. The two fragments of the PCR product, the kanamycin resistance cassette and pBluescript that had been digested with *Eco*RV were then

mixed and ligated. This was then transformed into *E. coli* with selection on ampicillin and kanamycin. Resultant clones were screened for the correct plasmid product. One of these was chosen for further studies. The entire insert from this plasmid was removed using *Pvu*II and cloned into the suicide vector pCVD442 which had been digested with *Sma*I. This was transformed into *E. coli* carrying the *pir* gene to allow pCVD442 to replicate. Resultant plasmids were again checked for the correct insert size. One of these was chosen to be used in making the mutant. *E. coli* donor bacteria were conjugated with *S. typhimurium* LB 5010 recipients using standard methods. After incubating the conjugation mixture, the bacteria were harvested and plated onto selective media containing kanamycin and sucrose. pCVD442 contains the *sacB* gene, the product of which confers sensitivity to the presence of sucrose in the medium. Plating on media containing sucrose thus selects against the presence of vector sequences. Of the colonies that grew on the selection plates, one was picked for further study. To check if the *msbB* gene has been mutated, chromosomal DNA was prepared and used as template in a PCR using the *msbB*-specific oligonucleotides. This showed a 2.3kb band in the mutant, with a 1 kb band appearing in the wild-type control, confirming that the mutant had a rearranged *msbB* gene. The LB 5010 strain is not virulent for mice. It serves as an intermediate in making mutants, since it is mutated in its DNA restriction system, but not its DNA modification system. DNA that passes through LB 5010 is thus modified, which allows a better frequency of introduction of the DNA into its final recipient. To move the chromosomal mutation from LB 5010 into the wild type virulent *S. typhimurium* strains, P22 transduction was used. Briefly, LB 5010 *msbB*::*Km* was infected with P22 HT101 *int* and plated. At the appropriate dilution confluent lysis was observed after overnight incubation at 37° C. These plates were harvested and the bacteriophage recovered as a plate stock. This stock was used to infect *S.*

typhimurium strains C5, SL1344 and HWSH with subsequent selection on kanamycin plates. Again, resulting colonies were checked for chromosomal rearrangement by PCR. The mutant bacteria gave a band at 2.3kb and the wild-type controls a band at 1 kb as expected, confirming that *msbB* mutants had been generated. Before biological assays were performed with these mutants, the LPS molecule was checked to see if the mutation affecting the lipid A domain had had any effects on the more distal parts of the molecule. An SDS-PAGE gel of the LPS from the wild-type and mutant bacteria was performed and stained with silver. No difference was observed between the LPS molecules in terms of ladder pattern of the O-antigen, or intensity of staining. Furthermore, the *msbB* mutant was also susceptible to infection with bacteriophage P22, which requires O-antigen for binding to the bacterial surface, confirming that the *msbB* mutants were able to synthesise full length LPS molecules.

Example 2 - Infection study in BALB/c mice

To test the virulence of the *S. typhimurium msbB* mutant it was injected into *nramp*-susceptible BALB/c mice and its growth *in vivo* was followed. Mice infected with wild-type organisms died as expected after 7 days of infection with counts in livers and spleens reaching approximately 10^8 per organ (Figure 1). Intriguingly, the *msbB* mutants grew at exactly the same rate as the wild-type (w.t.) bacteria, but only caused approximately 5% of the infected animals to die. Death only occurred when the bacterial counts had reached very high levels (approximately 10^9 per organ). After this, the mutant Salmonellae were gradually cleared until there were no counts in livers and spleens (Figure 1).

To investigate likely reasons why the mutant bacteria were not as lethal as their wild-type parents, cytokine release and iNOS induction *in vitro* and *in vivo* in response to the bacteria were measured. First the release of TNF-α and IL-1ß from J774 macrophage-like cells was measured. The cultured cells were incubated with 10^5 heat-killed wild-type or *msbB* mutant bacteria and the two cytokines were

measured in the culture medium by ELISA. It can be seen from Figure 2 that mutant bacteria induced 5-fold less TNF-α and half as much IL-1ß as their wild-type parents. This is as expected given that the *msbB* mutant has a reduced toxicity lipid A molecule. The release of NO from J774 cells was next determined. Again cultured cells were incubated with 10^7 heat-killed wild-type or *msbB* mutant bacteria and culture medium was assayed for NO by the Griess' reaction. The mutant bacteria induced half as much NO as the wild-type bacteria (Figure 3).

Finally, cytokine levels *in vivo* were measured. At 24 hours post-infection, serum samples were taken and assayed for TNF-α and IL-1ß using ELISA. These results show that the mutant induced 4-fold less TNF-α and half as much IL-1ß than the wild-type bacteria. These results correlate precisely with the *in vitro* results and strongly suggest that the reduced lethality of the *msbB* mutants is due to their reduced ability to induce potentially harmful cytokine responses: in short, because the toxicity of their lipid A molecule has been reduced. This is the first direct evidence that endotoxin is responsible for lethality in this infection.

Studies using the *msbB*, *aroA/msbB* and *aro* mutants of *S. typhimurium* - Vaccination and Challenge Studies

Example 3 - Oral Vaccination and Challenge

30 mice/group were inoculated with 10^8 or 10^9 oral by gavage tube *S. typhimurium aroA* or *S. typhimurium aroA/msbB*. Animals were left for 30 days and then challenged with wild type *S. typhimurium* 10^5 oral by gavage tube. Two separate groups of mice were inoculated as described above but animals in the two groups were killed on days 1, 3, 5, 9, 14, 21 and 28. The livers and spleens of infected mice were homogenised and viable counts performed on the surface of agar plates (see Figures 5 and 6).

Challenge with the wild type strain in the group receiving *S. typhimurium*

aroA resulted in all the animals surviving (100% protection, 30/30 mice alive). In the group given *S. typhimurium aroA/msbB* and challenged with *S. typhimurium* wild type 16/30 mice survived the wild type challenge (40% protection). This result indicates that the *aroA/msbB* mutation in *S. typhimurium* is more attenuated than the *aroA* mutation alone.

Example 4 - Intravenous Challenge of BALB/c mice with *S. typhimurium aroA* and *S. typhimurium msbB* (i.v. LD50).

Groups of mice were challenged intravenously with *S. typhimurium aroA* or *S. typhimurium msbB* at doses of 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9. Mice were left and observed for deaths in all groups. *S. typhimurium aroA* and *aroA/msbB* all lived at doses up to 10^6 i.v. All of both sets of mutants died at 10^7 i.v. but the *msbB* group lived significantly longer than the *aroA* infected group. *S. typhimurium aroA* infected mice died at day 7 but the *msbB* group lived up to 3 weeks after the *aroA* group died.

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Claims

1. Nucleic acid for a mutant *msbB* gene derivable from *Salmonella* which results in loss of an *msbB* encoded protein or loss of function of the protein, which in turn results in a lipid A molecule having reduced toxicity.

2. Nucleic acid for a mutant *htrB* gene derivable from *Salmonella* which results in loss of an *htrB* encoded protein or loss of function of the protein, which in turn results in a lipid A molecule having reduced toxicity.

3. Nucleic acid according to claim 1 or claim 2 wherein the lipid A molecule has reduced toxicity compared to a lipid A molecule resulting from a protein coded for by a wild-type gene.

4. Nucleic acid according to any preceding claim wherein the lipid A molecule is substantially non-toxic.

5. Nucleic acid according to any preceding claim wherein the lipid A molecule has one secondary acyl chain.

6. Nucleic acid according to any one of claims 1 to 4 wherein the lipid A molecule has no secondary acyl chains.

7. Nucleic acid according to any preceding claim wherein the mutant gene is not lethal for growth.

8. Nucleic acid according to any preceding claim wherein the lipid A molecule has a reduced ability to induce cytokines compared to a lipid A molecule resulting

from an MsbB protein coded for by a wild-type *msbB* gene.

9. Nucleic acid according to any preceding claim wherein the mutant gene is derivable or derived from *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Yersinia*, *Vibrio*, *Aeromonas*, *Pasteurella*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Flavobacterium*, *Bordetella*, *Actinobacillus*, *Neisseria*, *Brucella*, *Haemophilus* or *Escherichia coli*.

10. Nucleic acid according to claim 9 wherein the mutant gene is derived from *Salmonella*.

11. Nucleic acid according to claim 10 wherein the mutant gene is derivable or derived from *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella paratyphi* A or C, *Salmonella schottmulleri*, *Salmonella choleraesuis*, *Salmonella montevideo*, *Salmonella newport*, *Salmonella enteritidis*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella abortusovi*, *Salmonella abortus-equi*, *Salmonella dublin*, *Salmonella sofia*, *Salmonella havana*, *Salmonella bovis-morbificans*, *Salmonella hadar*, *Salmonella arizonae* or *Salmonella anatum*.

12. Nucleic acid according to any preceding claim derived by genetic manipulation of a *Salmonella msbB* or *htrB* gene comprising insertion of kanamycin resistance cassette to inactivate the gene, conjugation into a recipient to be mutated on a suicide vector, followed by P22 transduction into another recipient.

13. Nucleic acid for a mutant *msbB* gene having the characteristics of the mutant *msbB* gene contained in NCIMB Deposit Number 40856.

14. Isolated nucleic acid according to any preceding claim.

15. Nucleic acid according to any preceding claim which is cDNA or synthesised DNA.

16. A polypeptide which is coded for by the nucleic acid of any preceding claim.

17. A polypeptide according to claim 16 which is isolated from any other proteins with which it is naturally associated.

18. A recombinant DNA construct comprising the nucleic acid of any one of claims 1 to 15.

19. A recombinant DNA construct comprising the nucleic acid of any one of claims 1 to 15 cloned into a cloning or expression vector.

20. A recombinant micro-organism comprising the recombinant DNA construct of claim 18 or claim 19.

21. A recombinant micro-organism of claim 20 wherein the micro-organism is *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Yersinia*, *Vibrio*, *Aeromonas*, *Pasteurella*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Flavobacterium*, *Bordetella*, *Actinobacillus*, *Neisseria*, *Brucella*, *Haemophilus* or *Escherichia coli*.

22. A recombinant micro-organism of claim 21 wherein the micro-organism is *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella paratyphi* A or C, *Salmonella*

schottmulleri, *Salmonella choleraesuis*, *Salmonella montevideo*, *Salmonella newport*, *Salmonella enteritidis*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella abortusovi*, *Salmonella abortus-equi*, *Salmonella dublin*, *Salmonella sofia*, *Salmonella havana*, *Salmonella bovis-morbificans*, *Salmonella hadar*, *Salmonella arizonae* or *Salmonella anatum*.

23. A micro-organism having the characteristics of NCIMB Deposit Number 40856.

24. A micro-organism comprising nucleic acid for a mutant *msbB* gene derivable from *Salmonella* which results in loss of an *msbB* encoded protein or loss of function of the protein; an inactivated *msbB* gene; or which lacks an *msbB* gene, and having reduced toxicity.

25. A micro-organism according to claim 24 which is *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Yersinia*, *Vibrio*, *Aeromonas*, *Pasteurella*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Flavobacterium*, *Bordetella*, *Actinobacillus*, *Neisseria*, *Brucella*, *Haemophilus* or *Escherichia coli*, with the proviso that when the micro-organism is *Escherichia coli*, the mutant *msbB* gene is not from *Escherichia coli*.

26. A micro-organism comprising nucleic acid for a mutant *htrB* gene derivable from *Salmonella* which results in loss of an *htrB* encoded protein or loss of function of the protein; an inactivated *htrB* gene; or which lacks an *htrB* gene, and having reduced toxicity.

27. A micro-organism according to claim 26 which is *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Yersinia*, *Vibrio*, *Aeromonas*, *Pasteurella*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Flavobacterium*, *Bordetella*, *Actinobacillus*, *Neisseria*, *Brucella*, *Haemophilus* or *Escherichia coli*

28. A micro-organism comprising (i) nucleic acid for a mutant *msbB* gene derivable from *Salmonella* which results in loss of an *msbB* encoded protein or loss of function of the protein; an inactivated *msbB* gene; or which lacks an *msbB* gene, and (ii) nucleic acid for a mutant *htrB* gene derivable from *Salmonella* which results in loss of an *htrB* encoded protein or loss of function of the protein; an inactivated *htrB* gene; or which lacks an *htrB* gene, which in turn results in a lipid A molecule having reduced toxicity.

29. A micro-organism according to claim 28 which is *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Yersinia*, *Vibrio*, *Aeromonas*, *Pasteurella*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Flavobacterium*, *Bordetella*, *Actinobacillus*, *Neisseria*, *Brucella*, *Haemophilus* or *Escherichia coli*.

30. A micro-organism according to any one of claims 24 to 29 wherein the micro-organism is *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella paratyphi* A or C, *Salmonella schottmulleri*, *Salmonella choleraesuis*, *Salmonella montevideo*, *Salmonella newport*, *Salmonella enteritidis*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella abortusovi*, *Salmonella abortus-equi*, *Salmonella dublin*, *Salmonella sofia*, *Salmonella havana*, *Salmonella bovis-morbificans*, *Salmonella hadar*, *Salmonella arizonae* or *Salmonella anatum*.

31. A micro-organism according to any one of claims 24 to 30 which is substantially non-toxic.

32. A micro-organism according to any one of claims 24 to 31 in which the said nucleic acid is not conditionally lethal for growth.

33. A micro-organism according to any one of claims 24 to 32 which has a reduced ability to induce cytokines compared to a corresponding wild-type micro-organism.

34. A micro-organism according to any one of claims 24 to 33 which produces a lipid A molecule which has one secondary acyl chain.

35. A micro-organism according to any one of claims 24 to 33 which produces a lipid A molecule which does not have a secondary acyl chain.

36. A micro-organism according to any one of claims 24 to 35 comprising the nucleic acid of any one of claims 1 to 15 or in the form of a recombinant micro-organism of any one of claims 20 to 22.

37. A live vaccine comprising an attenuated or avirulent micro-organism comprising a mutant *msbB* gene, an inactivated *msbB* gene or lacking an *msbB* gene and which exhibits a reduced toxicity.

38. A live vaccine comprising an attenuated or avirulent micro-organism comprising a mutant *htrB* gene, an inactivated *htrB* gene or lacking an *htrB* gene and which exhibits a reduced toxicity.

39. A live vaccine comprising an attenuated or a virulent micro-organism comprising (i) a mutant ***msbB*** gene; an inactivated *msbB* gene; or lacking an ***msbB*** gene; and (ii) a mutant ***htrB*** gene; an inactivated *htrB* gene; or lacking an ***htrB*** gene, and which exhibits a reduced toxicity.

40. A vaccine according to any one of claims 37 to 39 wherein the micro-organism exhibits a reduced toxicity compared to a corresponding wild-type micro-organism.

41. A vaccine according to any one of claims 37 to 40 wherein the micro-organism is substantially non-toxic.

42. A vaccine according to any one of claims 37 to 41 wherein the mutated micro-organism is not lethal for growth.

43. A vaccine according to any one of claims 37 to 42 wherein the micro-organism produces a lipid A molecule which has one secondary acyl chain.

44. A vaccine according to any one of claims 37 to 42 wherein the micro-organism produces a lipid A molecule which does not have a secondary acyl chain.

45. A vaccine according to any one of claims 37 to 44 wherein the micro-organism is ***Salmonella***, ***Shigella***, *Klebsiella*, *Enterobacter*, *Serratia*, ***Proteus***, ***Yersinia***, ***Vibrio***, ***Aeromonas***, *Pasteurella*, *Pseudomonas*, *Acinetobacter*, ***Moraxella***, ***Flavobacterium***, ***Bordetella***, *Actinobacillus*, *Neisseria*, *Brucella*, *Haemophilus* or *Escherichia coli*.

46. A vaccine according to claim 45 wherein the micro-organism is *Salmonella typhimurium*, *Samonells typhi*, *Salmonella paratyphi* A or C, *Salmonella schottmulleri*, *Salmonella choleraesuis*, *Salmonella montevideo*, *Salmonella newport*, *Salmonella enteritidis*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella abortusovi*, *Salmonella abortus-equi*, *Salmonella dublin*, *Salmonella sofia*, *Salmonella havana*, *Salmonella bovis-morbificans*, *Salmonella hadar*, *Salmonella arizonae* or *Salmonella anatum*.

47. A vaccine according to any one of claims 37 to 46 derived from a micro-organism according to any one of claims 20 to 36, except that with reference to claim 25 the proviso does not apply.

48. A method for immunising a subject comprising administering an effective amount of a vaccine of any one of claims 37 to 47.

49. A pharmaceutical composition comprising a micro-organism according to any one of claims 20 to 36, except with reference to claim 25 the proviso does not apply.

50. Use of a micro-organism according to any one of claims 20 to 36, except with reference to claim 25 the proviso does not apply.

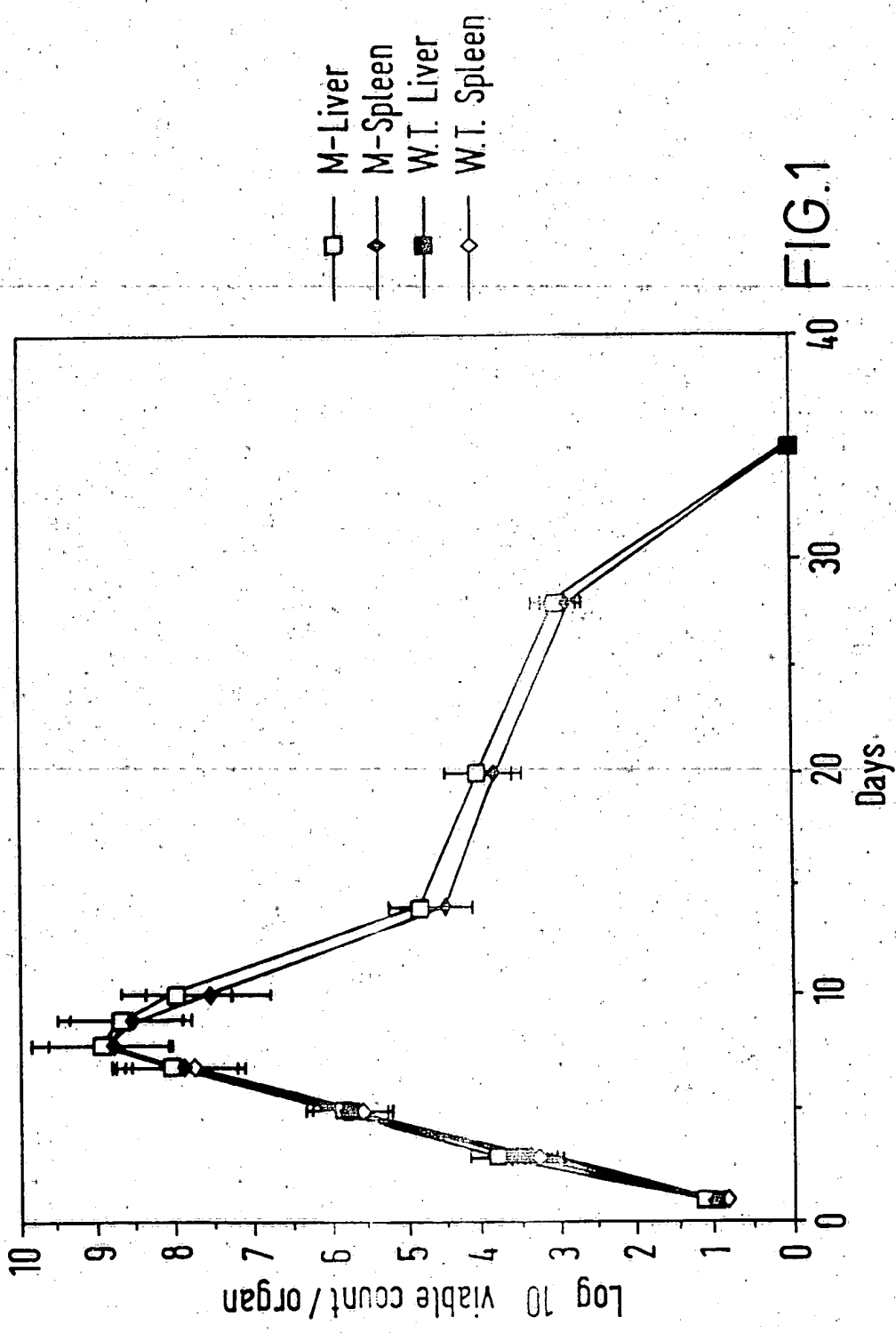

M-Liver
M-Spleen
W.T. Liver
W.T. Spleen
Log 10 viable count / organ
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40
Days

FIG.1

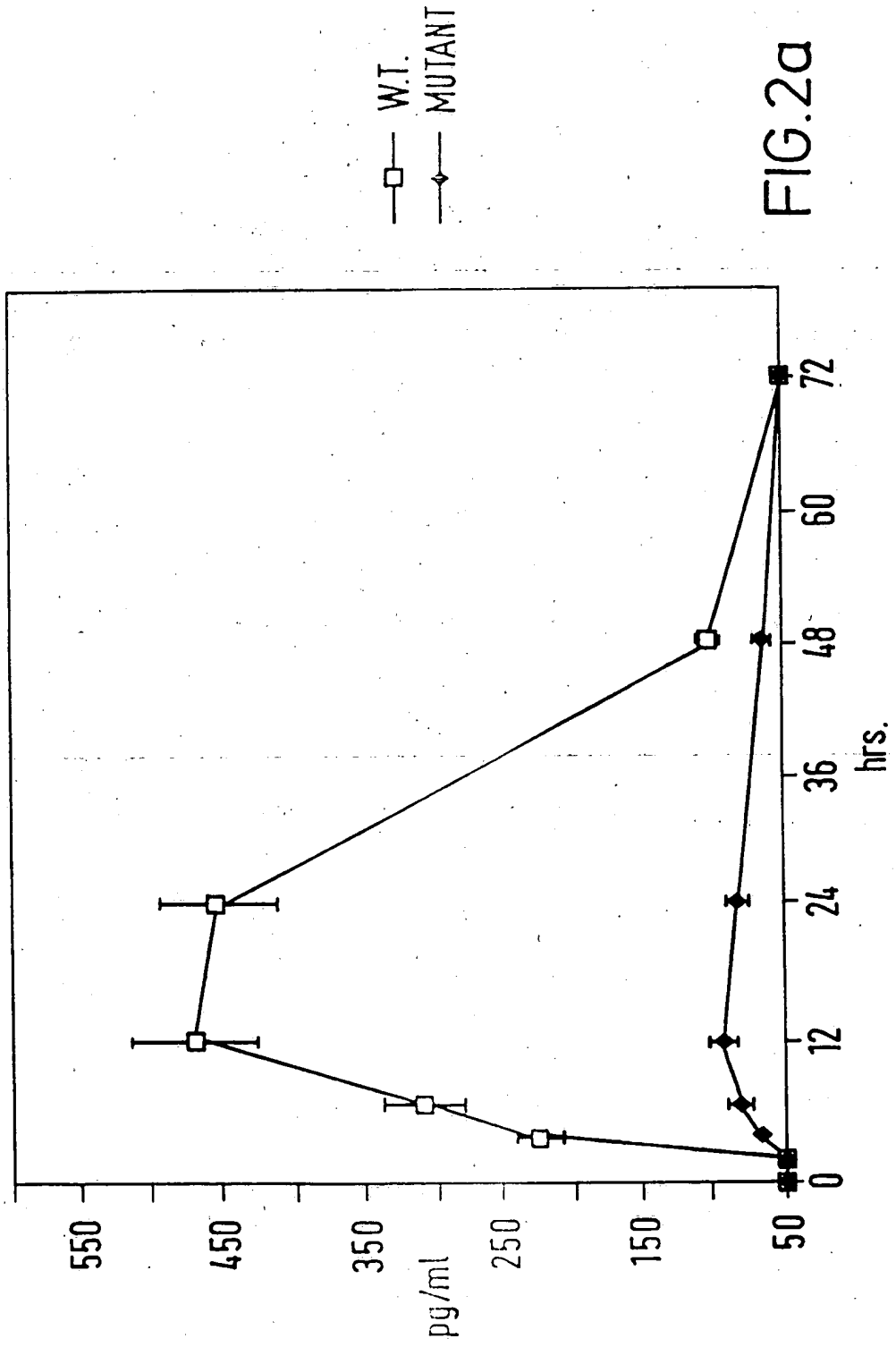


FIG.2a

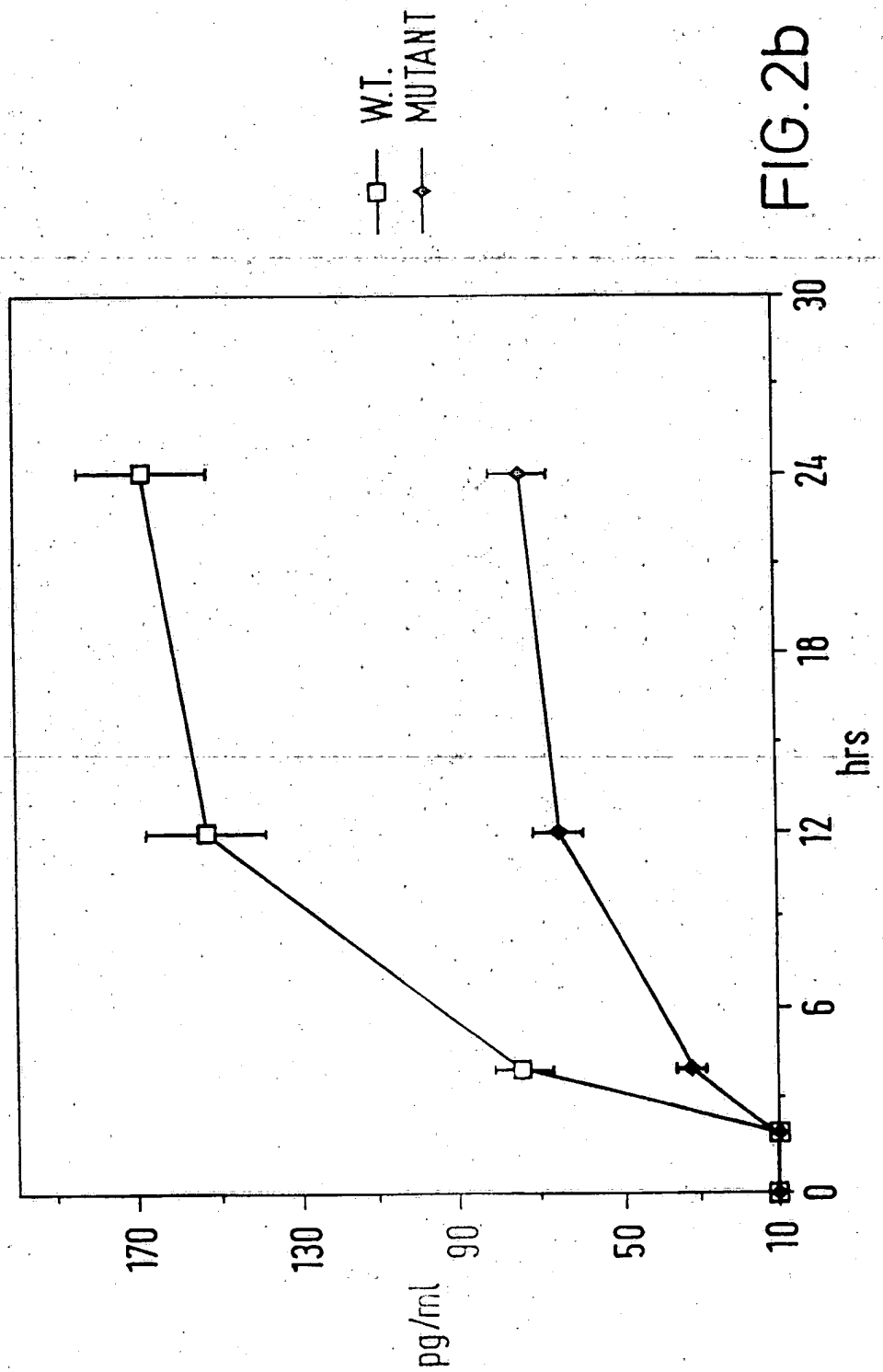


FIG. 2b

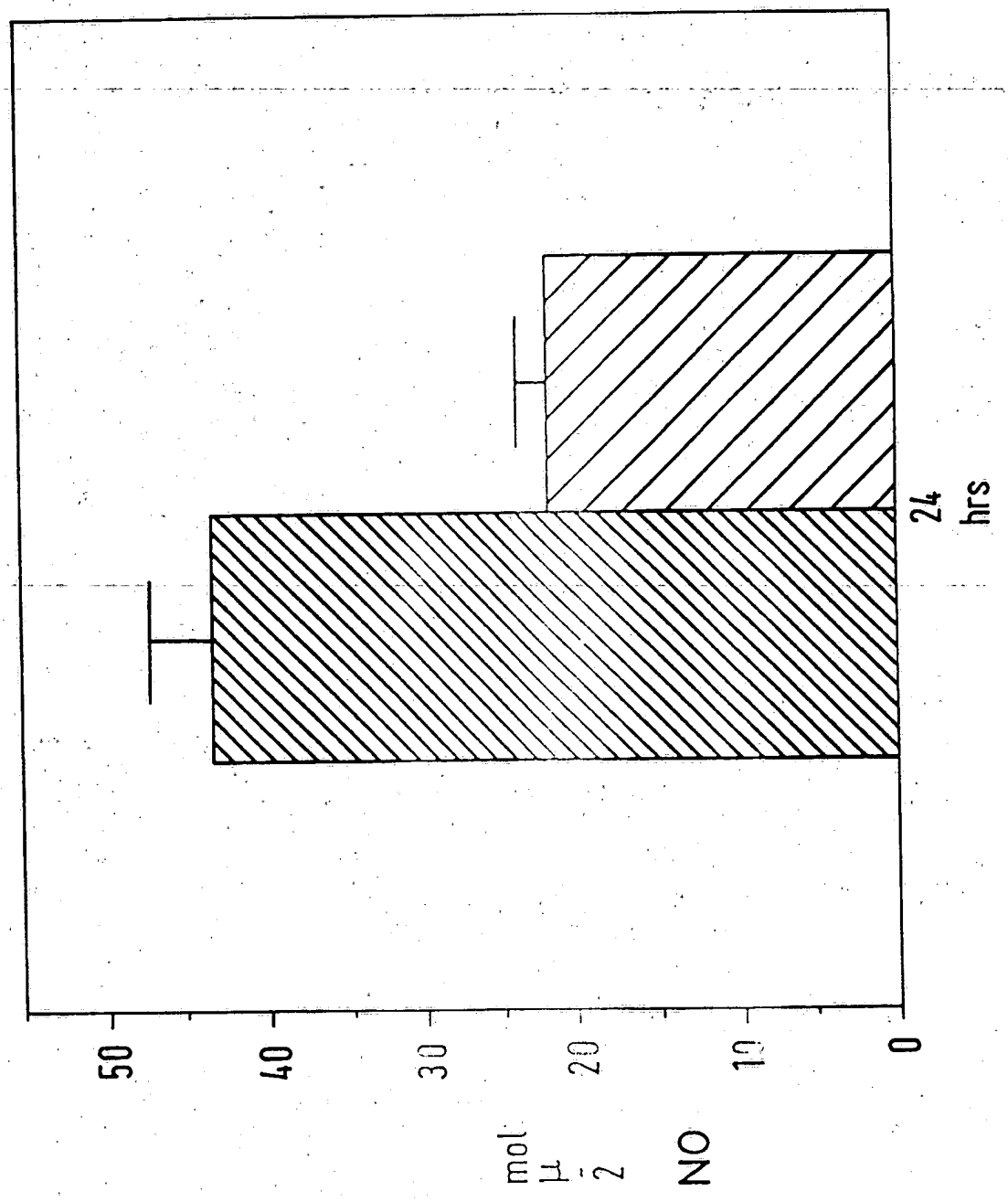
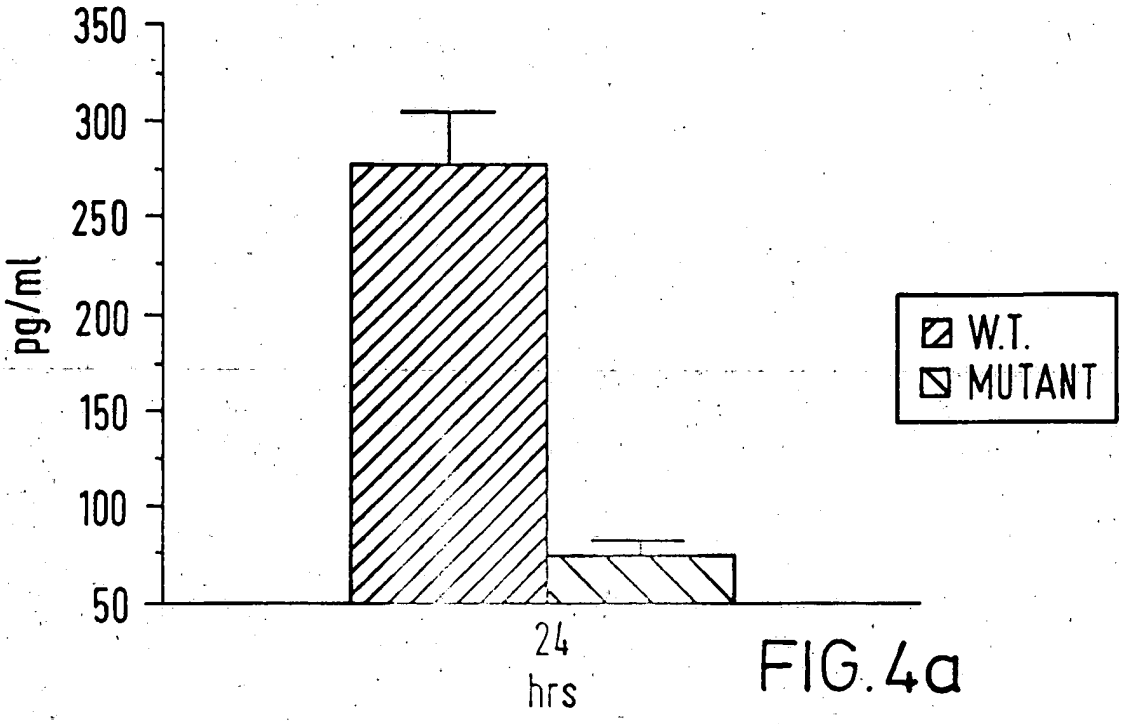


FIG.3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250
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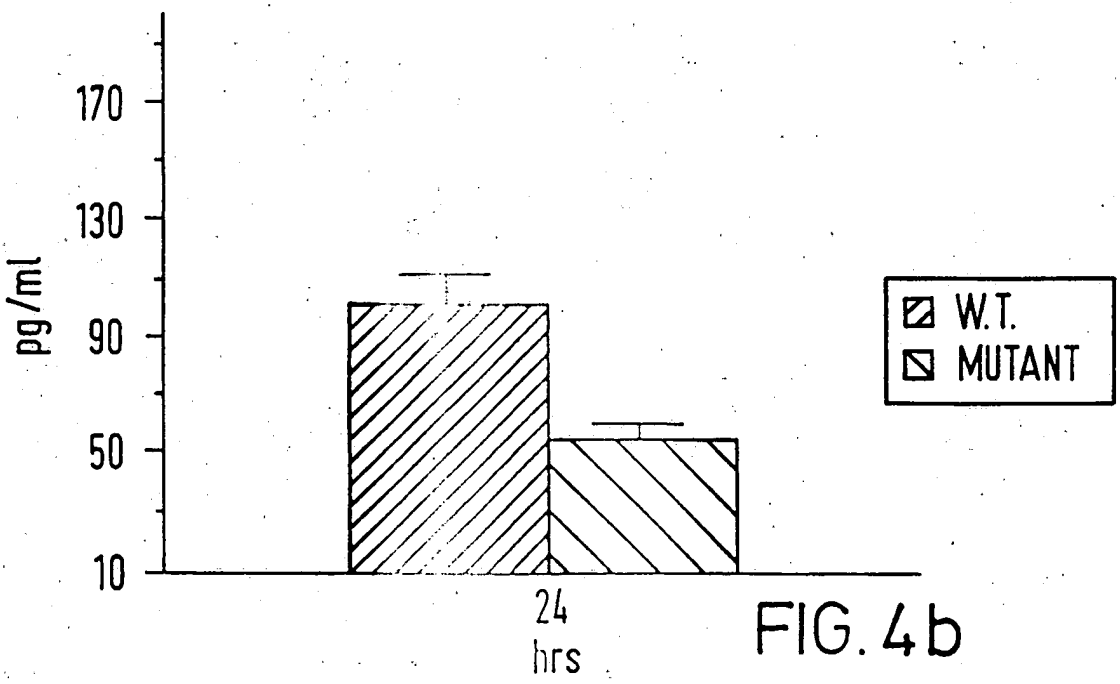
150
100
50
pg/ml
24
hrs
W.T.
MUTANT

FIG. 4a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hrs
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FIG. 4b

FIG.5

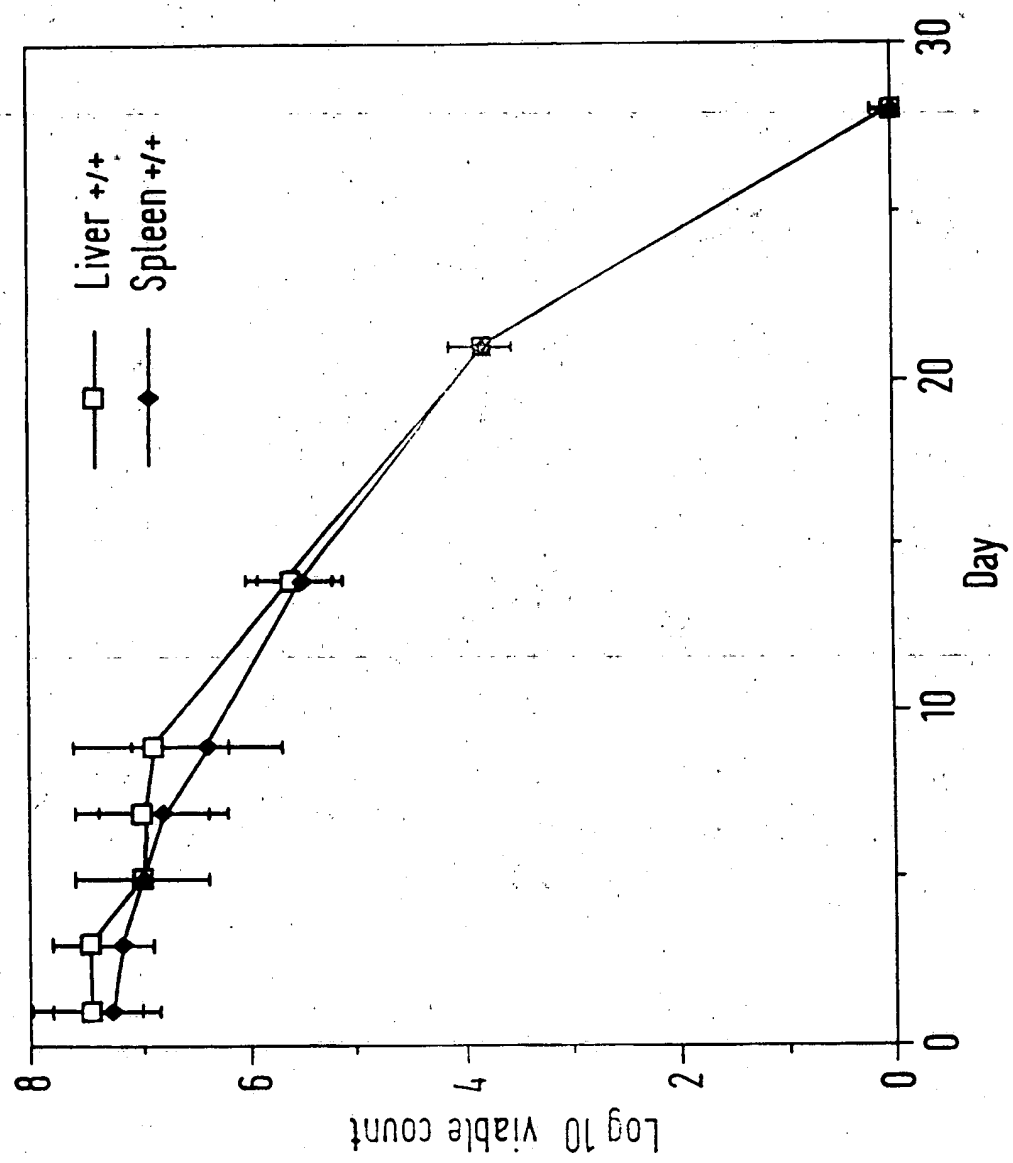
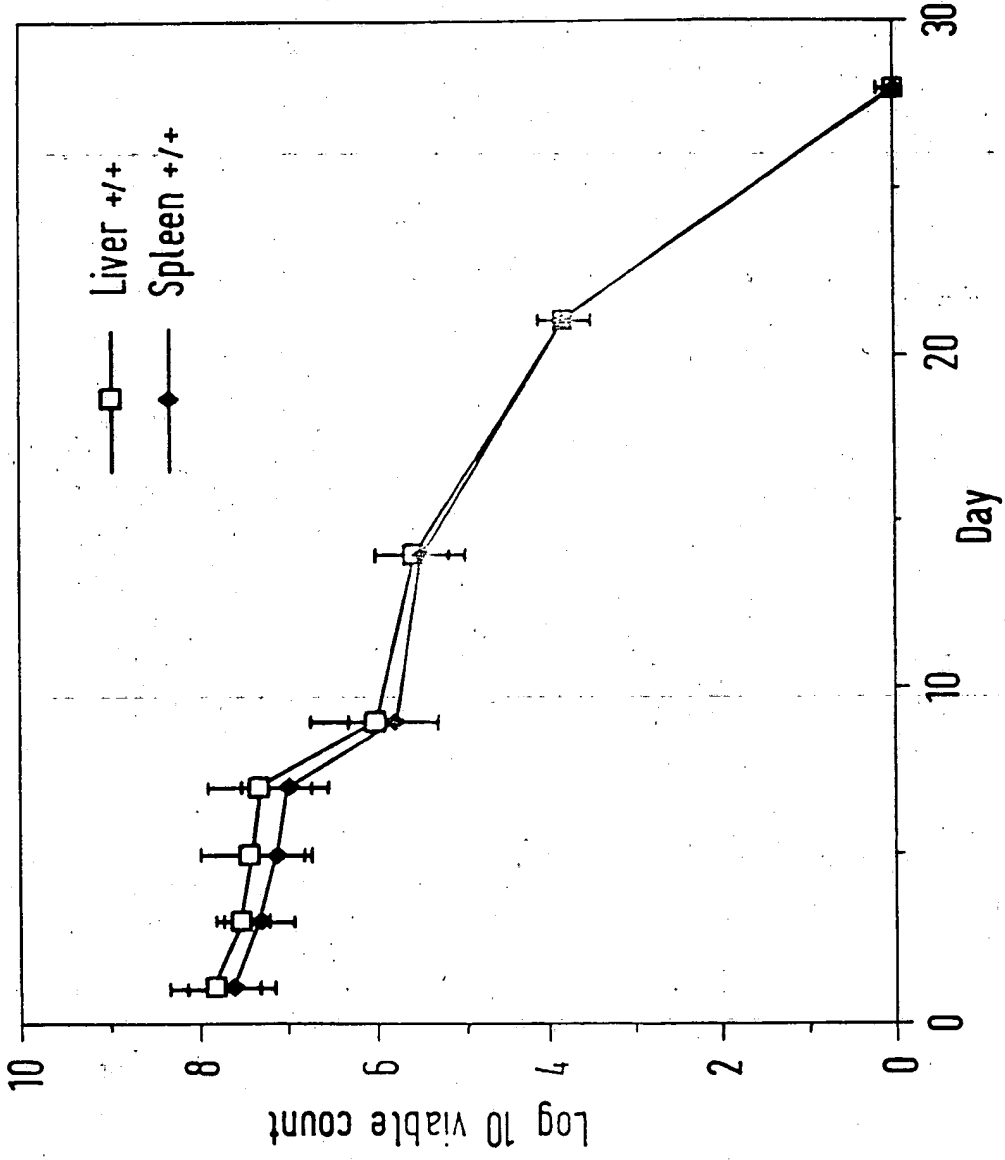


FIG.6



INTERNATIONAL SEARCH REPORT

Inte .ional Application No
PCT/GB 98/00291

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C07K14/195 C12N15/63 C12N1/20 A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SOMERVILLE J.E. ET AL.: "A novel Escherichia coli lipid A mutant that produces an antiinflammatory lipopolysaccharide" J. CLIN. INVEST., vol. 97, no. 2, January 1996, pages 359-365, XP002065990 see page 359, column 1 - column 2 see page 361, column 2, paragraph 4 see page 362, column 1, paragraph 4 - column 2, paragraph 1 --- -/--	1,3-9, 14,15, 17-21, 24,25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 May 1998	25/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chakravarty, A

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INTERNATIONAL SEARCH REPORT

Inte. ..onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	CLEMENTZ T. ET AL.: "Function of the htrB high temperature requirement gene of E. coli in the aclyation of lipid A" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 20, 17 May 1996, MD US, pages 12095-12102, XP002065992 see abstract see page 1208, column 1, paragraph 2 ---	2-5,7-9, 14,16, 18-21, 26, 31-34,36
P,X	WO 97 19688 A (UNIV IOWA RES FOUND ;UNIV CALIFORNIA (US); AMERICAN CYANAMID CO (U) 5 June 1997 see the whole document ---	2-12, 14-23, 26,27, 31-36, 38,40-50
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